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Supplemental Information

Interaction of Chk1 with Treslin Negatively Regulates the Initiation of Chromosomal DNA Replication

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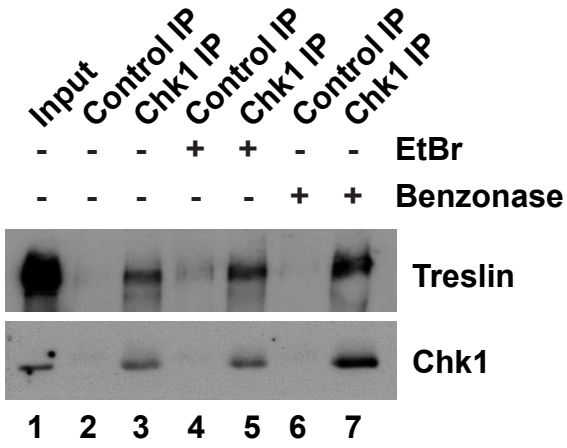
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Chk1 IP
Control IP
Chk1 IP
Control IP
Chk1 IP
- - - + + - - EtBr
- - - - - + + Benzonase
Treslin
Chk1
1 2 3 4 5 6 7

Figure S1

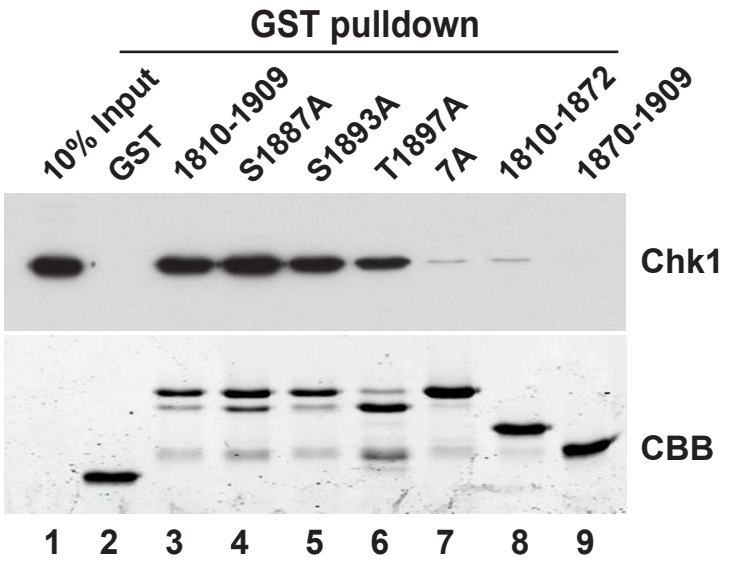
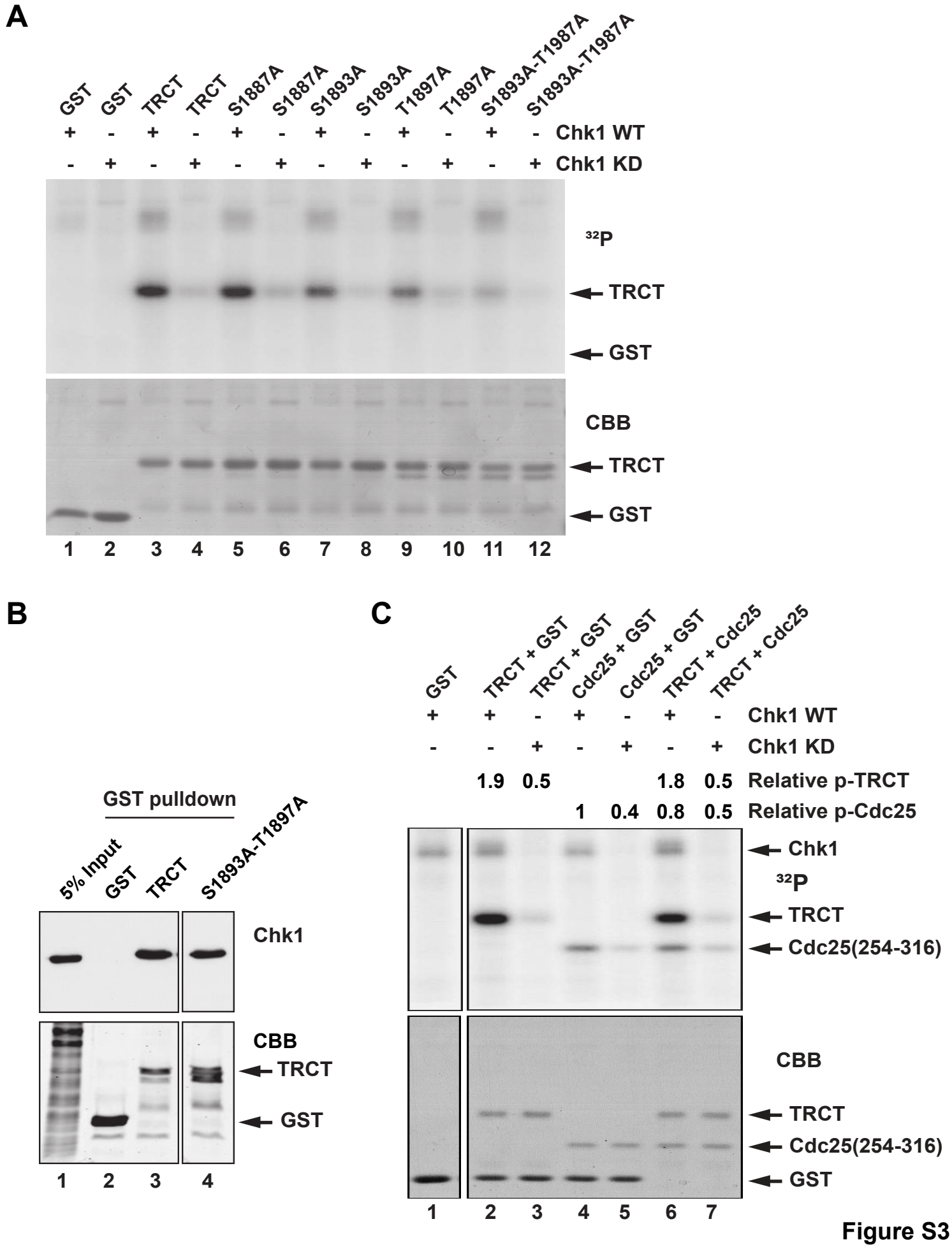

GST pulldown
10% Input
GST
1810-1909
S1887A
S1893A
T1897A
7A
1810-1872
1870-1909
Chk1
CBB
1 2 3 4 5 6 7 8 9

Figure S2


A
GST
GST
TRCT
TRCT
S1887A
S1887A
S1893A
S1893A
T1897A
T1897A
S1893A-T1987A
S1893A-T1987A
+ - + - + - + - + - + -
Chk1 WT
- + - + - + - + - + - +
Chk1 KD
32P
TRCT
GST
CBB
TRCT
GST
1 2 3 4 5 6 7 8 9 10 11 12
B
GST pulldown
5% Input
GST
TRCT
S1893A-T1897A
Chk1
CBB
TRCT
GST
1 2 3 4
C
GST
TRCT + GST
TRCT + GST
Cdc25 + GST
Cdc25 + GST
TRCT + Cdc25
TRCT + Cdc25
+ + - + - + -
Chk1 WT
- - + - + - +
Chk1 KD
1.9 0.5 1.8 0.5
Relative p-TRCT
1 0.4 0.8 0.5
Relative p-Cdc25
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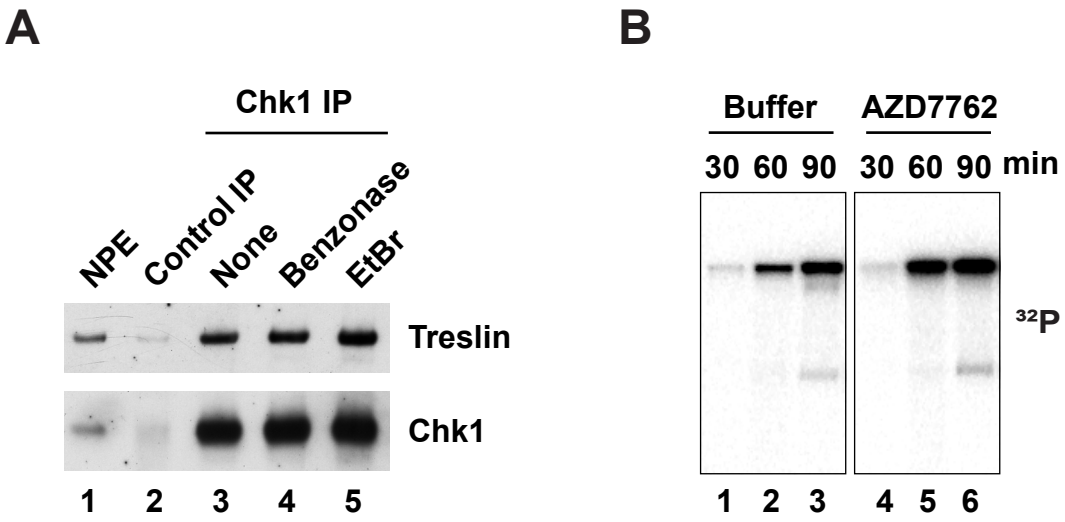
TRCT
Cdc25(254-316)
CBB
TRCT
Cdc25(254-316)
GST
1 2 3 4 5 6 7

Figure S3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Benzonase
EtBr
Treslin
Chk1
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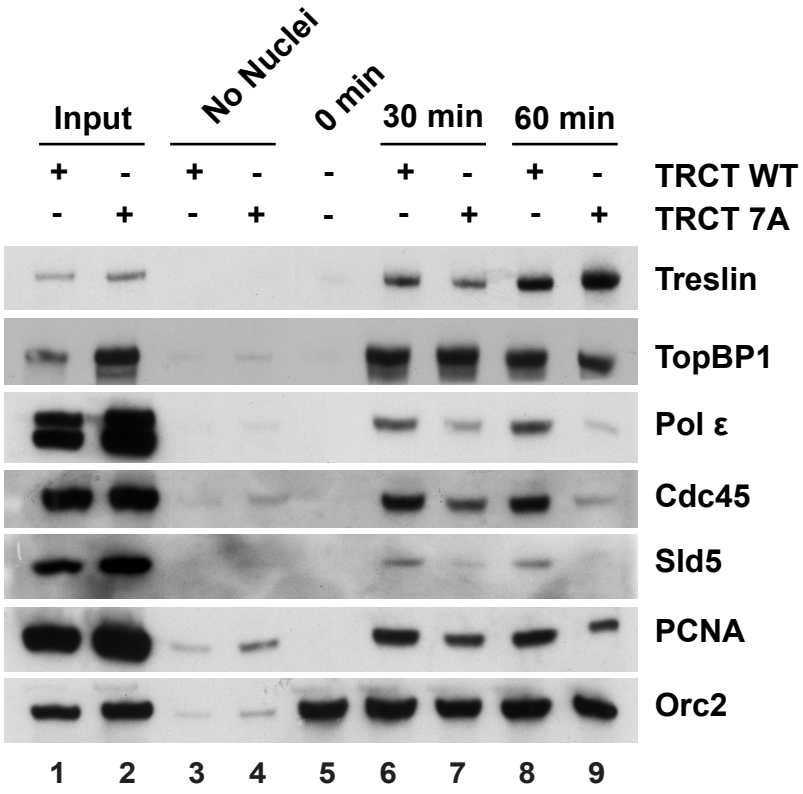
B
Buffer
AZD7762
30 60 90 30 60 90 min
32P
1 2 3 4 5 6

Figure S4


Input
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0 min
30 min
60 min
+ - + - - + - + -
- + - + - - + - +
TRCT WT
TRCT 7A
Treslin
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Pol ε
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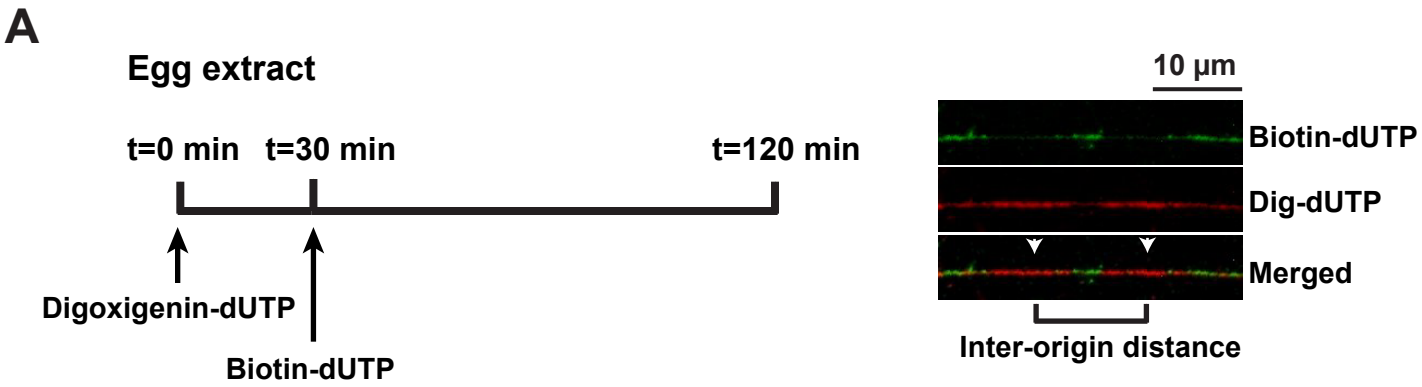
Sld5
PCNA
Orc2
1 2 3 4 5 6 7 8 9

Figure S5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Egg extract
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t=30 min
t=120 min
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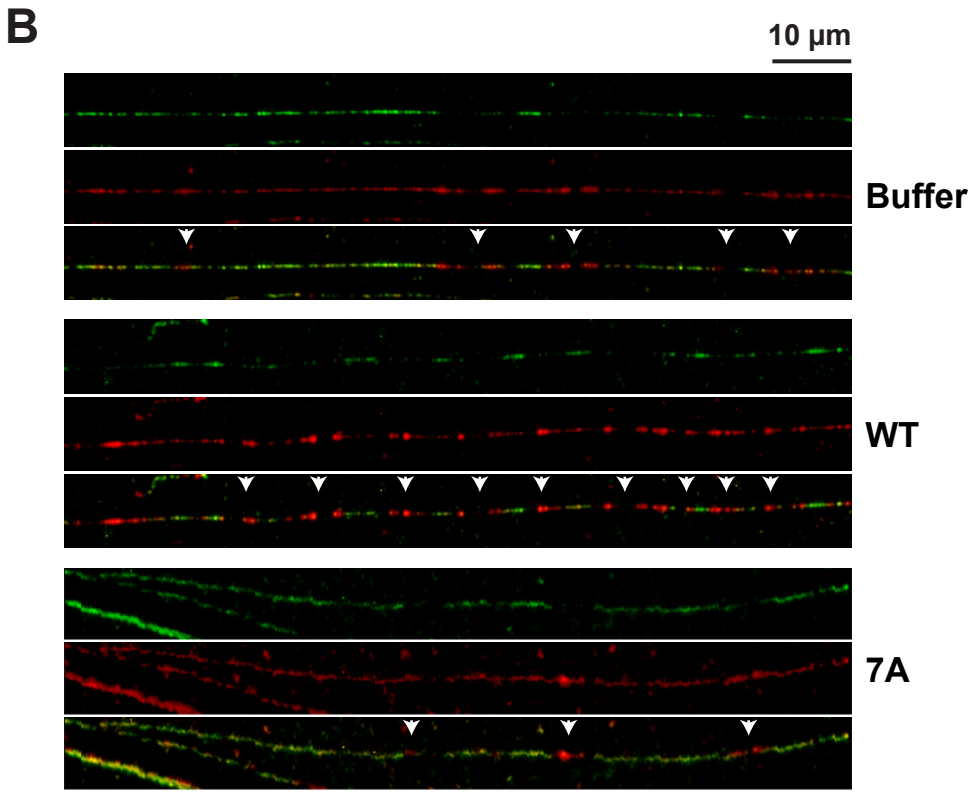
Biotin-dUTP
10 µm
Biotin-dUTP
Dig-dUTP
Merged
Inter-origin distance


B
10 µm
Buffer
WT
7A

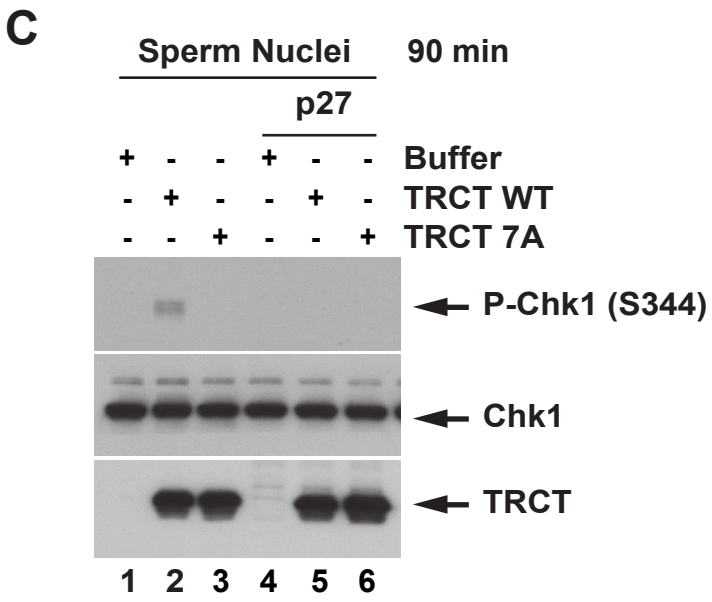

C
Sperm Nuclei
90 min
p27
+ - - + - - Buffer
- + - - + - TRCT WT
- - + - - + TRCT 7A
P-Chk1 (S344)
Chk1
TRCT
1 2 3 4 5 6

Figure S6

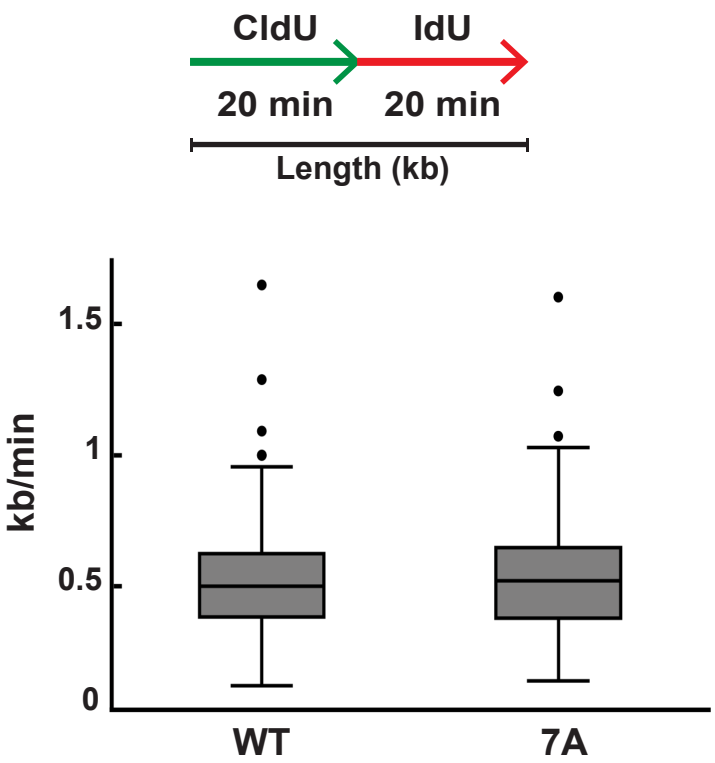


Figure S7

Figure S1. Treatment with Ethidium Bromide or Benzonase Does Not Inhibit Co-immunoprecipitation of Treslin and Chk1. Related to Figure 1.

293T nuclear lysates were treated with 50 μg/ml ethidium bromide (lanes 4 and 5) or 1 U/μl Benzonase (lanes 6 and 7) for 30 min on ice before immunoprecipitation was performed as in Figure 1C using control IgG (lanes 2, 4, and 6) or antibodies against Chk1 (lanes 3, 5, and 7).

Figure S2. The TRCT Domain Binds Directly to Chk1. Related to Figure 2.

GST only (lane 2), GST-tagged TRCT (lane 3), TRCT containing mutations S1887A (lane 4), S1893A (lane 5), T1897A (lane 6), or 7A (lane 7), and GST-tagged forms of residues 1810-1872 (lane 8) and 1870-1909 from Treslin (lane 9) were isolated from bacteria with glutathione agarose. Bead-bound proteins were incubated with recombinant His6-Chk1 in binding buffer at 4ºC for 90 min. Beads were retrieved, washed, and subjected to immunoblotting with anti-Chk1 (top) and staining with Coomassie blue (bottom). Recombinant Chk1 alone was loaded lane 1.

Figure S3. Further Characterization of the Phosphorylation of Treslin by Chk1. Related to Figure 3.

(A) GST only (lanes 1-2), GST-TRCT (lanes 3-4), and versions of this fragment containing the S1887A (lanes 5-6), S1893A (lanes 7-8), T1897A (lanes 9-10), or S1893A and T1897A mutations (lanes 11-12) were incubated with recombinant WT Chk1 (lanes 1, 3, 5, 7, 9, and 11) or KD Chk1 (lanes 2, 4, 6, 8, 10, and 12). Reactions were processed for phosphorimaging (top) and Coomassie blue staining (bottom).
(B) GST only (lane 2), GST-tagged form of wild-type TRCT (lane 3), and a version of this fragment containing both the S1893A and T1897A mutations (lane 4) were expressed in bacteria and isolated with glutathione agarose. Bead-bound fragments were incubated with 293T nuclear lysates. Beads were retrieved, washed, and processed for immunoblotting with anti-Chk1 (top) and Coomassie blue staining (bottom). 293T nuclear lysate was loaded in lane 1 as input.
(C) GST only (lane 1), GST-TRCT plus GST (lanes 2-3), GST-Cdc25(254-316) plus GST (lanes 4-5), and GST-TRCT plus GST-Cdc25(254-316) (lanes 6-7) were incubated with recombinant WT Chk1 (lanes 1, 2, 4, and 6) or KD Chk1 (lanes 3, 5, and 7). Reactions were processed for phosphorimaging (top) and Coomassie blue staining (bottom). Relative levels of phosphorylation normalized to protein amount are shown. Phosphorylation of Cdc25(254-316) in lane 4 was denoted as 1.

Figure S4. Further Characterization of the Effect of Chk1 on DNA Replication. Related to Figure 4.

(A) NPE was treated with 1 U/μl Benzonase (lane 4) or 50 μg/ml ethidium bromide (lane 5) for 30 min on ice before immunoprecipitation was performed as in Figure 4D.
(B) HSS was incubated for 30 min at room temperature. NPE containing buffer (lanes 1-3), or AZD7762 (lanes 4-6) was added to HSS. The final concentration of AZD7762 was 0.5 μM.

Samples were incubated in the presence of [α-^{32}P]dATP to determine chromosomal DNA replication. Quantitation of the results is presented in Figure 4E.

Figure S5. The TRCT Domain Stimulates the Loading of Numerous Replication Proteins onto Chromatin. Related to Figure 6.

HSS was incubated without (lanes 3-4) or with sperm nuclei (lanes 5-9) for 30 min. NPE and either WT (lanes 1, 3, 6, and 8) or 7A TRCT (lanes 2, 4, 7, and 9) at a final concentration of 60 ng/µl were added to HSS. Chromatin was isolated at indicated times and immunoblotted with the indicated antibodies. NPE/HSS mixture was loaded in lanes 1-2.

Figure S6. Determination of Inter-origin Distances in Egg Extracts Treated with the TRCT Domain. Related to Figure 6.

(A) Schematic of DNA fiber assay in *Xenopus* egg extracts and fiber visualization by immunofluorescence.
(B) Representative images of fibers from egg extracts containing buffer alone, TRCT WT (75 ng/µl), or TRCT 7A (75 ng/µl) are shown. Distributions of inter-origin distances are shown in Figure 6C.
(C) HSS was incubated in the presence of sperm nuclei (lanes 1-6) for 30 min. NPE either lacking (lanes 1-3) or containing 1 µM GST-p27 (lanes 4-6) and buffer (lanes 1 and 4), TRCT WT (lanes 2 and 5), or TRCT 7A (lanes 3 and 6) were added to HSS. Final concentration of TRCT was 60 ng/µl. Mixtures were incubated for 90 min and immunoblotted with anti-P-Chk1 (top), anti-Chk1 (middle), and anti-GST (bottom).

Figure S7. Replication-fork Speed in Cells Expressing WT or 7A Treslin. Related to Figure 7.

U2OS T-Rex cells harboring Treslin WT or 7A were cultured in the presence of doxycycline for 48 hr. DNA fibers were prepared and replication-fork speed was determined.

Table S1. Full list of proteins purified specifically with the Treslin 1253-1909 C-terminal fragment. Related to Figure 1.

N	IPI acc no	Protein name	Pulldown performed with Treslin			Control Pulldown[c] [84][b]
			Full-length[a] [484][b]	1253-1909[a] [104][b]	1-1257 [518][b]	
1	IPI00301395	Probable serine carboxypeptidase CPVL	5/5	13/43	-	-
2	IPI00023664	Serine/threonine-protein kinase Chk1	9/13	10/23	-	-
3	IPI00470610	Pyrroline-5-carboxylate reductase 2	5/6	7/18	-	-
4	IPI00012578	Importin subunit alpha-4	2/8	3/15	-	-
5	IPI00299033	Importin subunit alpha-3	6/8	6/11	-	-
6	IPI00879374	Metastasis-associated protein MTA1	2/3	6/7	-	-
7	IPI00941557	Pyrroline-5-carboxylate reductase 1	2/4	2/7	-	-
8	IPI00640085	Isoform 1 of E3 ubiquitin-protein ligase UBR2	3/4	5/6	-	-
9	IPI00171798	MTA2 metastasis-associated 1 family	3/4	2/5	-	-
10	IPI00021536	CALML5 calmodulin-like 5	2/2	2/4	-	-
	IPI00413144	Treslin	94/1252	48/2447	73/1381	-

[a] exclusive unique peptide count/total spectrum count reported by Scaffold
[b] total number of proteins identified in pulldown as reported by Scaffold under settings described in the Experimental Procedures section
[c] control pulldown from cells containing vector alone

SUPPLEMENTAL EXPERIMENTAL PROCEDURES

Plasmids

Deletion mutants were created by PCR-based methods. Point mutations were created with the QuikChange kit using Pfu DNA polymerase (Agilent Technologies). All mutations were confirmed by DNA sequencing.

Additional Antibodies

Anti-FLAG (M2) and anti-human TopBP1 (A300-111A) antibodies were obtained from Agilent Technologies and Bethyl Laboratories, respectively. An anti-human mouse monoclonal antibody that also recognizes *Xenopus* PCNA and anti-human Orc2 antibodies were purchased from BD Biosciences. Anti-human-phospho-Chk1 antibodies (S345, 133D3), which also recognize S344-phosphorylated *Xenopus* Chk1, were purchased from Cell Signaling Technology. Antibodies against human Chk1 (G-4) and GST (Z-5) were obtained from Santa Cruz Biotechnology. Antibodies against *Xenopus* Chk1, TopBP1, Cdc45, p60 of DNA polymerase epsilon, and Orc2 were described previously (Kumagai et al., 1998; Kumagai et al., 2006; Lee et al., 2005; Lee et al., 2003). Antisera against *Xenopus* Sld5 were generously supplied by H. Takisawa (Osaka University, Osaka, Japan). Antibodies against *Xenopus* ISWI were prepared as described (Demeret et al., 2002). An anti-human Mcm2 mouse monoclonal antibody (BM28) that cross-reacts with *Xenopus* Mcm2 was purchased from EM Biosciences. For the DNA fiber assays, anti-CldU (anti-BrdU BU1/75(ICR1)) and anti-IdU antibodies (anti-BrdU, clone B44) were obtained from Novus Biologicals and BD Biosciences, respectively. Alexa Fluor 594 goat anti-mouse and Alexa Fluor 488 donkey anti-rat antibodies were purchased from Invitrogen. NeutrAvidin Oregon Green 488 was purchased from Life Technologies.

Mouse anti-digoxigenin Alexa Fluor 594 was purchased from Jackson ImmunoResearch. Biotinylated anti-avidin was obtained from Thermo Scientific.

Protein Purification and Mass Spectrometry Analysis

Plasmids encoding different versions of Treslin-SF were each transfected into a total of six 15-cm dishes of 293T cells. DNA (17 μg) was first diluted in 2.5 ml OPTI-MEM, which was then supplemented with 20 μg/ml polyethyleneimine. After 15 min, the mixture was added to each dish. After 48 hr, cells were harvested and lysed in 5.4 ml hypotonic buffer (10 mM Hepes-KOH, pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM DTT, 0.5 mM PMSF, and 0.6 μM tautomycin) for 10 min on ice. NP-40 was added to 0.05% and lysates were centrifuged for 10 min at 330 g at 4ºC. The pellet was resuspended in 6.75 ml high salt buffer containing 20 mM Hepes-KOH, pH 7.5, 500 mM NaCl, 0.5% Triton X-100, 5 mM EDTA, 1 mM DTT, 10 mM β-glycerolphosphate, 1 mM NaF, 0.1 mM vanadate, 0.6 μM tautomycin, 1 mM PMSF, 10 μg/ml each of pepstatin, chymostatin, and leupeptin. The supernatant was collected by centrifugation at 16,000 g for 10 min and diluted with 20 mM Hepes-KOH (pH 7.5) to 150 mM NaCl. The supernatant was again collected by centrifugation at 16,000g for 5 min and incubated with 180 μl anti-FLAG M2 agarose (Sigma) at 4ºC for 4 hr. After washing, Treslin-associated proteins were eluted overnight in 0.9 ml elution buffer containing 20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.5 mM EDTA, 1 mM DTT, 0.5 mM PMSF, and 120 ng/μl 3X-FLAG peptide. The eluate was collected by centrifugation and the anti-FLAG resin was eluted twice more with 1.35 ml 20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.13% Triton-X-100, and 0.5 mM PMSF. All eluates were combined and incubated with 260 μl S protein agarose at 4ºC for 4 hr. The samples were washed with 20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.1% Triton-X-100, and 0.5 mM PMSF and eluted in SDS sample buffer.

The Treslin-associated proteins were resolved by SDS-PAGE, stained with Coomassie, and subjected to GeLC MS/MS analysis. Briefly, complete gel lanes were sliced into 6-8 slabs, each of which was *in-gel* digested with trypsin (Shevchenko et al., 2006). Tryptic peptides recovered from the gel matrix were subjected to LC-MS/MS at the Ultimate 3000 nanoLC system (Thermo Dionex, Idstein, Germany) interfaced to a LTQ Orbitrap Velos hybrid mass spectrometer (Thermo Fisher Scientific, Bremen, Germany) as described (Vasilj et al., 2012). The acquired spectra were searched against an IPI Human sequence database (version 3.86) using Mascot software (MatrixScience, London, UK) under the following settings: tryptic enzyme specificity; mass tolerance of 5 ppm and 0.5 Da for precursor and fragment ions, respectively; up to two mis-cleavages allowed; variable modifications: oxidation of methionine; deamidation of glutamines and asparagines; propionamidation of cysteines, protein acetylation at the N-terminus; and, where specified, serine / threonine / tyrosine phosphorylation. Protein hits were further validated using Scaffold software (Proteome Software, Portland, USA, version 2.04) under the following settings: two or more matched peptides required under 95% and 99% peptide and protein statistical significance thresholds, respectively. False Discovery Rate (FDR) as calculated by the Scaffold software was below 0.1.

Preparation and Processing of Nuclear Lysates from Human Cells

Cells were lysed in high salt buffer containing 20 mM Hepes-KOH, pH 7.5, 500 mM NaCl, 0.5% Triton X-100, 5 mM EDTA, 1 mM DTT, 10 mM β-glycerolphosphate, 1 mM NaF, 0.1 mM vanadate, 0.6 μM tautomycin, 1 mM PMSF, 10 μg/ml each of pepstatin, chymostatin, and leupeptin. The supernatant was collected by centrifugation at 16,000 g for 10 min and diluted with 20 mM Hepes-KOH (pH 7.5) to 150 mM NaCl. The supernatant was again collected by centrifugation at 16,000 g for 5 min.

For pulldowns or immunoprecipitations, lysates were incubated for 2 hr at 4ºC with S protein agarose, anti-FLAG agarose, or antibodies bound to protein A or protein G magnetic beads. The samples were washed four times with 20 mM Hepes-KOH, pH 7.5, 150 mM NaCl, 0.15% Triton X-100, 1.5 mM EDTA, 1 mM DTT, 10 mM β-glycerolphosphate, 1 mM NaF, and 0.1 mM vanadate. For kinase assays with Treslin-SF proteins, anti-FLAG beads were collected, washed twice with buffer containing 20 mM Hepes-KOH, pH 7.5, 150 mM NaCl, 0.15% Triton X-100, 1.5 mM EDTA, and 0.3 mM DTT; twice with high salt buffer containing 20 mM Hepes-KOH, pH 7.5, 450 mM NaCl, and 1 mM DTT; twice with RIPA buffer containing 20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 2 mM EDTA, 1% NP40, and 0.1% SDS; and twice with HBS buffer containing 20 mM Hepes-KOH, pH 7.5, and 150 mM NaCl. After washing, bead-bound proteins were either stored at -80˚C or used immediately.

Production of GST Fusion Proteins

Plasmids encoding GST-tagged versions of the TRCT were introduced into *Escherichia coli* Rosetta (DE3)pLysS cells (EMD). *E. coli* cells expressing the fragments were resuspended in 0.2 M sodium borate, pH 8.2, containing 0.5 M NaCl, 0.5% NP-40, 5 mM EGTA, and 1 mM PMSF, and then were sonicated. The lysates were clarified by centrifugation for 10 min at 20,000 g and incubated with glutathione agarose at 4ºC for 2 hr. The proteins were eluted with 50 mM Tris-HCl (pH 8.0) containing 10 mM reduced glutathione. Typically, the expressed proteins were dialyzed into ELB. GST-p27 was purified as described previously (Kumagai et al., 2011).

In Vitro Direct Binding Assay

GST-tagged human Treslin fragments were expressed in bacteria and isolated with glutathione agarose beads. Bead-bound fragments (1 μg) were incubated with 200 ng His6-

tagged *Xenopus* Chk1 (Kumagai et al., 1998) in 0.5 ml of binding buffer containing 20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.5% NP40, 1 mM EDTA, 1 mM DTT, and 10% glycerol at 4ºC for 90 min. The beads were retrieved, washed, and subjected to immunoblotting and staining with Coomassie brilliant blue.

Kinase Assays

Full-length human Treslin-SF proteins (WT and 7A) bound to anti-FLAG beads or soluble GST-tagged human TRCT fragments were incubated with WT or KD (N135A) *Xenopus* His6-Chk1 (Kumagai et al., 1998) in 40 µl of kinase buffer containing 10 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 1 mM DTT, 2 µCi [γ-^{32}P] ATP, and 10 µM ATP at room temperature for 30 min. Reactions were terminated with SDS sample buffer. For assays with Cdc25, we utilized a GST-tagged version of residues 254-316 from *Xenopus* Cdc25 in which S287 is the sole site of phosphorylation for Chk1 (Kumagai et al., 1998).

Use of the *Xenopus* NPE System

HSS (33 µl) was mixed with 1.32 µl ATP regeneration mix (10 µl 1 M phosphocreatine, 10 µl 100 mM ATP, and 0.5 µl 5 mg/ml creatine kinase), 0.5 µl 0.2 mg/ml nocodazole, and sperm nuclei (4,000 or 10,000 per µl for replication and chromatin-binding assays, respectively). At 15 min, 20 µl NPE was diluted with 46 µl ELB and supplemented with 2.64 µl ATP regeneration mix and 2.64 µl 50 mM DTT. For replication assays, 5 µCi [α-^{32}P]dATP was also added to NPE. At 30 min, TRCT fragments or AZD7762 were added to the HSS. One volume of HSS was then immediately mixed with two volumes of NPE and incubated as indicated. For gel analysis of reactions, incubations were terminated with SDS sample buffer. For preparation of chromatin fractions, reactions were mixed with 60 µl ice cold ELB containing 0.2% Triton X-

100 and transferred onto 180 µl ELB containing 0.5 M sucrose. Chromatin was collected by centrifugation at 16,000 g and washed twice with ELB containing 0.25 M sucrose. For replication assays, aliquots from reactions were mixed with an equal volume of 2X stop solution (2% SDS, 75 mM EDTA, 25 mM Tris-HCl, pH 8) and digested with proteinase K. Samples were electrophoresed in 0.8% agarose gels. Gels were dried and subjected to phosphorimaging.

Preparation of Nuclear Lysates from Egg Extracts

Methods have been described previously (Kumagai et al., 2010). In brief, egg extracts containing 3,000 demembranated sperm nuclei per µl were incubated at room temperature for 90 min. Nuclei were isolated by centrifugation for 5 min at 6,100 g through a sucrose cushion (1M sucrose dissolved in 20 mM Hepes-KOH, pH 7.5, 80 mM KCl, 2.5 mM potassium gluconate, and 10 mM magnesium gluconate). Pellets were resuspended in the sucrose solution and centrifuged again to collect nuclei. The nuclei were resuspended in 450 mM NaCl dissolved in 20 mM Hepes-KOH, pH 7.5, 10 mM β-glycerolphosphate, 6 mM DTT, 2.5 mM EGTA, 1 mM NaF, 0.1 mM sodium vanadate, 0.6 µM tautomycin, 0.1% NP-40, 0.1 mM PMSF, and 10 µg/ml each of pepstatin, chymostatin, and leupeptin. The lysates were centrifuged at 11,000 g for 10 min to remove particulate material and chromatin. The supernatant was diluted with 20 mM Hepes-KOH (pH 7.5) to adjust the concentration of NaCl to 75 mM and centrifuged again. The salt-extracted nuclear lysates were subject to SDS-PAGE and immunoblotting.

DNA Replication Assays with Whole Egg Extracts

For replication assays in whole egg extracts, 1 µCi [α-^{32}P]dATP was added to 10 µl egg extract containing 0.4 mM $CaCl_2$ and 3,000 sperm nuclei per µl. At indicated time points, reactions were stopped by addition of 10 µl 2X sample buffer (80 mM Tris-HCl, pH 8.0, 8 mM

EDTA, 0.13% phosphoric acid, 10% Ficoll, 5% SDS and 0.2% bromophenol blue). Samples were digested with 1 mg/ml proteinase K at 37ºC for 1 hr and loaded onto gels containing 1% agarose. Gels were dried and subjected to phosphorimaging.

DNA Fiber Assays with *Xenopus* Egg Extracts

Methods were described previously with some modifications (Bellelli et al., 2014; Marheineke et al., 2009). Sperm nuclei (3000/µl) were incubated in egg extracts containing 20 µM digoxigenin-11-dUTP (Roche) in the presence of buffer, TRCT WT (75 ng/µl), or TRCT 7A (75 ng/µl). At 30 min, biotin-16-dUTP (Biotium) was added to extracts at a final concentration 20 µM. At 120 min, nuclei were isolated by centrifugation for 5 min at 6,100 g through a sucrose cushion containing 1M sucrose dissolved in 20 mM HEPES-KOH, pH 7.5, 80 mM KCl, 2.5 mM potassium gluconate, and 10 mM magnesium gluconate. Pellets were resuspended gently in sucrose cushion and centrifuged again to collect nuclei. Nuclei were resuspended in PBS. The nuclear suspension was spotted onto one end of a microscope slide (Fisher Scientific) and mixed with six volumes of lysis buffer (0.5% SDS, 50 mM EDTA, 200 mM Tris-HCl, pH 7.4) for 10 min. The slide was carefully tilted 15º to allow the fibers to spread by gravity. Fibers were then air dried, fixed in methanol and acetic acid (3:1) for 3 min, and subsequently denatured with 2 N HCl for 1 hr. Slides were neutralized and washed with PBS (once at pH 8.0 and 3 times at pH 7.4), and blocked with 1% nucleic acid blocking reagent (Roche) in PBS at room temperature for 1 hr. Slides were incubated with NeutrAvidin Oregon Green 488 (1/50) and mouse anti-digoxigenin Alexa Fluor 594 (1/50) in blocking buffer at room temperature for 1 hr. Slides were washed and incubated with biotinylated anti-avidin and goat anti-mouse Alexa Fluor 594 in blocking buffer at room temperature for 1 hr. Slides were again washed and incubated with NeutrAvidin Oregon Green 488 (1/50) in blocking buffer for 45 min. Slides were

washed and mounted in Vectashield mounting medium (Vector Laboratories) prior to analysis using an Axioplan microscope (Carl Zeiss) equipped with a Plan Neofluar (63x/0.75 NA) objective. Inter-origin distances were measured using ImageJ software (National Institutes of Health; http://rsbweb.nih.gov/ij/) and kb values were obtained by multiplying micrometers by 2.59 (Jackson and Pombo, 1998).

DNA Fiber Assays with Human Cells

After labeling with CldU and IdU, cells were harvested and resuspended in cold PBS. The cell suspension was spotted onto one end of a microscope slide (Fisher Scientific) and mixed with six volumes of lysis buffer (0.5% SDS, 50 mM EDTA, 200 mM Tris-HCl, pH 7.4) for 5 min. The slide was carefully tilted 15º to allow the fibers to spread by gravity. Fibers were then fixed in methanol and acetic acid (3:1) for 3 min and subsequently denatured with 2 N HCl for 1 hr. Slides were neutralized and washed with PBS (once at pH 8.0 and 3 times at pH 7.4) and blocked with 10% goat serum and 0.1% Triton-X in PBS for 1 hr. Slides were incubated with primary antibodies against IdU (1:100 in blocking buffer) and CldU (1:200 in blocking buffer) and secondary antibodies (Alexa Fluor 488 donkey anti-rat and Alexa Fluor 594 goat anti-mouse, 1:300 in blocking buffer) for 1 hr each. Slides were mounted in Vectashield mounting medium (Vector Laboratories) prior to analysis using an Axioplan microscope (Carl Zeiss) equipped with a Plan Neofluar (63x/0.75 NA) objective.

SUPPLEMENTAL REFERENCES

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